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14. ABSTRACT

Despite that the level of TGFβ is increased locally and systemically in advanced breast tumors particularly at the leading edges and in metastasis, the molecular basis of a role of TGF β in metastatic breast cancer remained elusive. Our studies demonstrate that over-expression of *SMAD7* localized to chromosome 18q is frequently observed in breast tumors providing an alternate mechanism for the disruption of Smad signaling pathway in breast cancer. The inactivation of Smad signaling due to overexpression of *SMAD7* in cell culture was associated with differential expression of genes that favor epithelial to mesenchymal transition (EMT). We believe that these observations suggest that intact Smad signaling plays a major role in the suppression of breast cancer metastatic phenotype. In the future studies, we are planning to use model cell lines to identify and characterize the mediator and effector genes that regulate metastatic progression of breast cancer upon inactivation of the Smad signaling mediated events.

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Table of Contents

ANNUAL REPORT OF THE USAMRMC FUNDED ACTIVITY

Title of the grant: Metastatic progression of breast cancer by allelic loss on chromosome 18q21.

1. Introduction/ Project Overview/ Scientific Progress and future directions:

An association has been established between the high frequency of deletion of chromosome 18q21, where the *SMAD4* gene is localized, with advanced stages of cancers (1). These observations have received added credence from a recent report that suggested up-regulation of metastasis mediator genes such as VEGF and down-regulation of metastatic suppressor genes such as TSP1 in cell lines with *SMAD4* deletions or mutation (2). Our preliminary data suggest that inactivation of Smad signaling in breast cancer is primarily due to over-expression of Smad7 rather than due to mutational inactivations of the *SMAD* genes such as *SMAD4* or *SMAD2* as frequently observed in colon and pancreatic cancers. Furthermore, our preliminary data also provides the first direct evidence that over-expression of Smad7 leads to suppression of anti-angiogenic/ metastatic factors such as TSP-1. The ability of HER2/Neu, a well established breast cancer marker for advanced tumors, to enhance expression of *SMAD7* observed by others is also consistent with inactivation of Smad signaling in advanced breast cancer *via* this mechanism (3).

Based on these observations, we hypothesize that the simultaneous over-expression of Smad7 and elevated levels of TGF-beta enables the breast cancer cells to acquire pro-oncogenic properties in promoting advanced metastatic breast cancer. Here, we have outlined a strategy to test our hypothesis using cell culture model systems and to exploit high throughput methods to identify critical players in advanced metastatic breast cancer. We predict that these genes could serve as ideal candidates for tumor markers for staging, prognosis and as nodal points for therapeutic intervention of this devastating disease.

2. Modified tasks, summary of findings and future directions:

We are making steady progress in increasing the understanding of the implications of the direct or indirect inactivation of Smad signaling events due to overexpression of *SMAD7* localized to chromosome 18q in metastatic breast cancer. Although our original hypothesis was based on similarities between breast and gastrointestinal cancers in Smad signaling inactivation during cancer metastasis, the mechanism of inactivation in breast cancer appears to be due to overexpression of Smad7 rather than due to mutational inactivation of Smad4. Thus, the Career Development Award from the BCRP of the Department of Defense has enabled us to make significant progress towards deciphering this very important molecular basis for breast cancer metastasis. However, we are acutely aware of the critical task and the efforts ahead of us in completing this project. We hope to obtain additional funding from either the Department of Defense or other agencies to achieve these goals.

Task# 1: Development of cell culture model systems for over-expression of Smad7 and identifying factors involved in metastatic breast cancer.

Breast cancer cell culture model systems will be accessed if available or developed from cell lines representing breast epithelial cells which are either normal or cancerous representing different stages to study the promotion of angiogenesis/ metastasis due to over-expression of Smad7 and elevated levels of TGF-beta. We also plan to determine the requirement for precursor lesions for the effects of over-expression of Smad7 and elevated levels of TGF-beta causing conversion to metastatic phenotype. With the help of the model cell lines, we hope to identify differentially expressed genes responsible for breast cancer metastasis using cDNA microarrays.

Task# 2: Characterization of the metastasis regulator, mediator and effector genes resulting from Smad7 and TGF-beta over-expression.

We plan to catalogue the functional properties of candidate genes of interest predicted to be involved in breast cancer metastasis from the studies described in task#1. We will begin to build the frame-work for an expandable breast cancer metastasis gene network using the pre-existing data or derived using bioinformatics tools. These analyses will be followed up with limited phenotypic, cellular, behavioral and biochemical characterization of candidate genes in tumors and cell cultures to confirm or exclude their potential involvement in breast cancer metastasis.

Task# 3: Evaluation of potential therapeutic targets for breast cancer angiogenesis/ metastasis using the cell culture model systems.

We hope to conduct the preliminary experiments for selected potential therapeutic target genes identified in tasks 1 and 2 for their suitability for clinical trial considerations. Our major strategy is to use either RNA interference (siRNA) or inhibitor treatment to inhibit the effects of candidate genes and score for reversion of angiogenic/ metastatic effects using cell culture and potentially mouse xenograft models.

Body: Procedures and progress report:

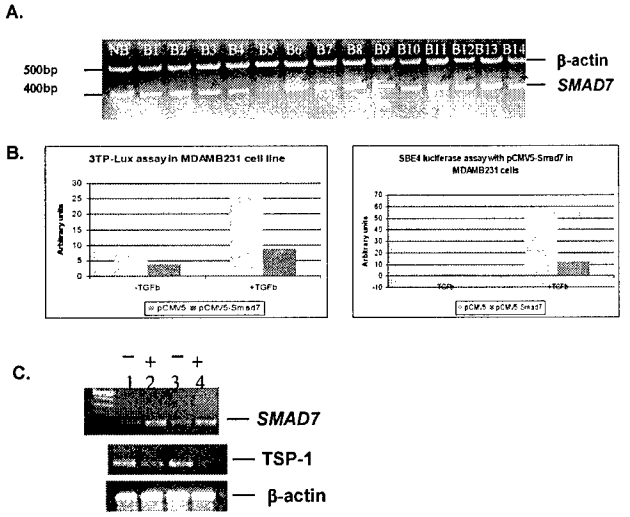

Figure 1. The levels of Smad7 in breast cancer and its effect on overall Smad signaling and expression of effector genes.

A. Total RNA was prepared using the Trizol method from the indicated breast cancer cell lines and specimens and analyzed by RT-PCR. The lanes NB represent normal tissue sample of the breast. The samples 2-8 are cell lines and 9-14 are tumor samples of the breast (lane 1). The red arrows indicate samples with SMAD7 over-expression. B. The effect of Smad7 overexpression on the Luciferase reporter with the 3TP or SBE4 promoters in the presence/ absence of TGF-beta in MDAMB231. C.The indicated genes were analyzed by semi-quantitative RT-PCR (right hand panel) in a cell line transiently transfected with a constitutively SMAD7 expressing (+) plasmid or its corresponding vector control (-).

Although overexpression of TGF-beta is widely observed in advanced breast cancers, disruption of Smad signaling in breast cancer cannot be explained on the basis of mutational inactivation directly affecting the *SMAD* genes or receptors for TGF-beta as they are rare events (1, 4). Therefore, we considered the possibility that the inactivation of the Smad signaling pathway could occur at an alternate point leading to the same end effects. in breast cancer. We examined over-expression of Smad7 as an alternative mechanism to achieve similar effects. Because of the lack of Smad7 specific monoclonal antibodies that give clean results we decided to use RT-PCR analysis to evaluate *SMAD7* expression. Our study showed that over-expression of *SMAD7* is a frequent occurrence in breast cancer (Figure 1A). We also confirmed the disruptive effect of Smad7 over-expression on TGF-beta signaling at the level of gene regulation by performing reporter assays (Figure 1B). Furthermore, our preliminary data was also consistent with the hypothesis that over-expression of Smad7 could promote the suppression in the expression of anti-angiogeneic/ metastatic factors such as TSP-1 (Figure 1C). These observations prompted us to test a hypothesis that "elevated levels of both Smad7 and TGF-beta play a critical role in metastatic conversion of advanced breast cancer" using appropriate normal breast and breast cancer cell line model systems.

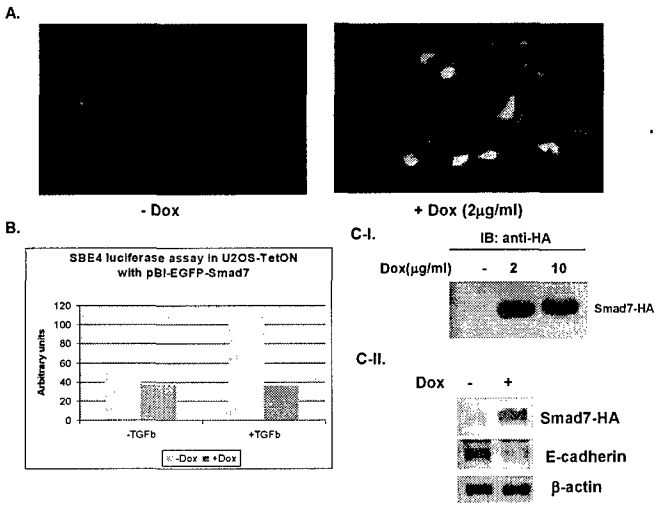

Figure 2: Establishment of a Tet-responsive inducible system in order to study the effect of Smad7 over-expression in breast cancer progression. *A. Addition of doxycyclin allows the inducible expression of both GFP (A.) by immunofluoresence microscopy and HA-tagged Smad7 by Western blotting (C-I.) in a bidirectional TetON promoter as evaluated in the COS7 cells. B. The inducible expression of Smad7 can mediate inhibition of the TGFβ-dependent Smad signaling pathway. C-II. The over-expression of Smad7 causes suppression in the expression of the epithelial marker gene, E-Cadherin as analyzed by Western blotting.*

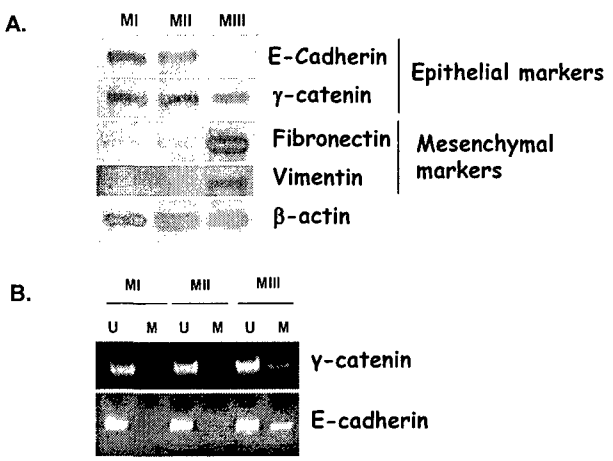

Figure 3. Progression from normal to early carcinoma is associated with increasing tendency for expression of EMT marker genes in a breast cancer progression model. *A. Western blotting analysis for the indicated epithelial and mesenchymal markers was used to evaluate the breast cancer model cell lines that represent the various stages of cancer progression. Lanes 1-3 correspond to derivatives of the MCF10A cell lines MI, MII and MIII. B. MSP analysis of the γ-catenin and E-cadherin promoters.*

As an initial step, we have generated an inducible tet-responsive Smad7 expression system in the pBI-

EGFP plasmid. Thus, we are able to turn on and off Smad7 expression and track the cells expressing Smad7 due to the bi-directional green fluorescence protein (GFP) expression (Figure 2A). The functionality of the HA-Smad7 similar to untagged Smad7 is also confirmed by Smad-responsive luciferase reporter assays (Figure 2B). Furthermore, we also found that expression of Smad7, could suppress an epithelial marker, E-Cadherin (Figure 2C-II). This is consistent with the idea that over-expression of Smad7 may be associated with the tendency to undergo epithelial to mesenchymal transition (EMT).

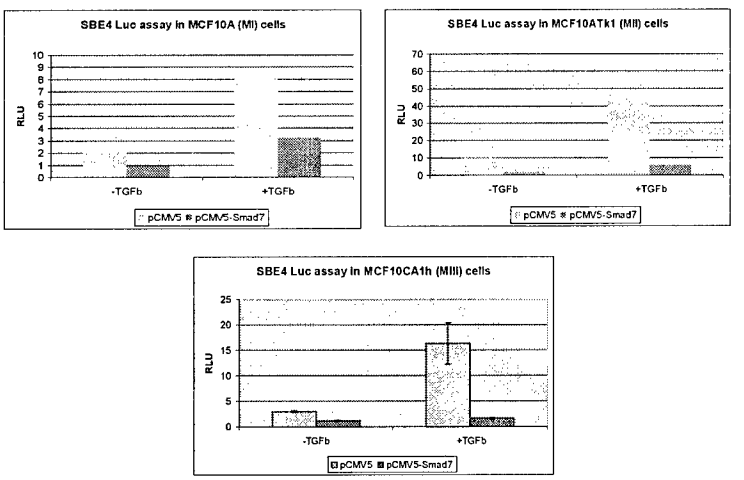

Figure 4. The effect of Smad7 overexpression on TGF-beta/ Smad signaling in the MI, MII and MIII cells.

The over-expression of Smad7 causes inhibition of TGF-beta/ Smad signaling in the MCF10A derivative cell lines MI, MII and MIII respectively as determined using the SBE4- luciferase reporter.

We are planning to use different groups of basal model cell lines with the following characteristics for our experiments: (1) breast cancer cell lines that exhibit normal levels of Smad7 expression; (2) breast cancer cell lines that exhibit elevated levels of Smad7 expression; (3) model cell lines derived from MCF10A that represent the normal epithelium (MI), and premalignant hyperplastic epithelium (MII) and low-grade carcinoma of the breast (MIII) (5). The breast cancer cell lines with different levels of *SMAD7* expression were selected based on our analyses as shown in Figure 1A. Our rationale for using the MI, MII and MIII cell lines is to determine the tumor stage specificity of Smad7 over-expression as a player in the conversion of benign to metastatic breast cancer. As an initial step, we decided to characterize these cell lines (i.e., MI, MII and MIII) at the marker gene expression levels to determine whether they truly represent different stages of breast cancer by Western blotting analysis for the known epithelial and mesenchymal markers (Figure 3A; 5). It is noteworthy that these cell lines have not been previously characterized for expression of epithelial and mesenchymal markers.

These studies strongly supported a strong correlation between a tendency for loss of expression of epithelial markers and accompanying up-regulation of mesenchymal markers in the model cell line that represent an advanced stage (MIII) while the cell lines representing early tumor stages (i.e., MI and MII)

showed expression of only epithelial markers (Figure 3A). Furthermore, with the aid of methylation specific PCR (MSP) analysis, we also demonstrate that down regulation of gamma-catenin and E-cadherin expression is likely to be due to epigenetic promoter DNA methylation in the MIII cells (Figure 3B). These studies suggest that genetic and epigenetic alterations can modulate differential gene expression driving the tumor cells from one to the other stage during breast cancer progression. In order to determine the intactness of TGF-beta/ Smad signaling and the ability of Smad7 over-expression to inhibit the pathway in MI, MII and MIII cell lines, we performed SBE4-luciferase reporter assays (Figure 4). These analyses showed that the pathway is intact and our Smad7 expression construct is functional in the model cell lines.

We believe that our preliminary studies strongly support a potential role for Smad7 over-expression is likely to be involved in advanced metastatic breast cancer. Additionally, we have already acquired and verified the suitability of the necessary basal model cell lines and the reagents to test this hypothesis.

Although our studies described here alone may not lead to the whole picture of molecular details for breast cancer metastasis, it is nevertheless highly likely to make major contributions towards the formulation of a "metastatic breast cancer network".

4. Key research accomplishments:

Our study has so far provided the important clues to understand that the molecular basis of Smad signaling inactivation is likely to be predominantly due to overexpression of Smad7 rather than mutations in the *SMAD4* gene in breast cancer.

We have also identified appropriate tumor cell lines as well as experimentally generated derivative test and control cell lines as model systems to identify and isolate the metastatic breast cancer mediator and effector genes that are expressed as a result of the inactivation of the Smad signaling pathway.

5. Conclusions:

(1) The inactivation of the Smad signaling pathway in breast cancers is primarily mediated by overexpression of Smad7.

(2) The identification of metastatic breast cancer mediator and effector genes using the cell culture model systems could potentially provide not only novel and valuable prognostic tumor markers but could also serve as key targets to combat breast cancer.

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7. Scientific presentation/ publications/ patent relevant to this grant:

Presentations by Dr. Sam Thiagalingam:

The Smad connection to cancer, Session Co-Chair, 9th World Congress on Advances in Oncology and 7th International Symposium on Molecular Medicine, Crete, Greece - October 14-16, 2004

A Multi-Modular Molecular Network Model for Cancer, Biomolecular Seminar Series, Boston University (Charles River campus) - April 4, 2005

The Smad8 connection to breast cancer, Era of Hope-2005, Philadelphia, PA - June 8-11, 2005

Cascade of Modules of a Network Define Cancer Progression, Session Chair, IXth Technological Advances in Science, Medicine and Engineering Conference and Workshop 2005 Quelph, Canada, July 9, 2005

Publications:

1. Thiagalingam, S., K-h.Cheng, H. J. Lee, N. Mineva, and J. F. Ponte. 2003. Histone deacetylases: Unique players in shaping the epigenetic histone code, *Annal. New York Acad. Sci.* **983**: 86-100.

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3. Papageorgis, P., K-h. Cheng, J. F. Ponte and S. Thiagalingam. 2005. Smad signaling inactivation promotes cancer metastasis. *Manuscript in preparation.*

Patent:

1. Method of determining gene expression-Targeted Expressed Gene Display - PCT Number: US0409143 (05/09/05); Boston University.